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Overexpression of copper zinc superoxide dismutase impairs human trophoblast cell fusion and differentiation

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SUMMARY

The syncytiotrophoblast (ST) is the major component of the human placenta, involved in feto-maternal exchanges and secretion of pregnancy-specific hormones. Multinucleated ST arises from fusion of mononuclear overexpression cytotrophoblast cells. In Trisomy 21-affected placentas, we recently have shown that there is a defect in ST formation and a decrease in the production of pregnancy-specific hormones. Due to the role of oxygen free radicals in trophoblast cell differentiation, we investigated the role of the key antioxidant enzyme copper/zinc superoxide dismutase (SOD-1), encoded by chromosome 21, in *in vitro* trophoblast differentiation. We first observed that over-expression of SOD-1 in normal cytotrophoblasts impaired ST formation. This was associated with a significant decrease in mRNA transcript levels and secretion of hCG and of other hormonal markers of ST. We confirmed abnormal cell fusion by overexpression of GFP-tagged SOD-1 in cytotrophoblasts. In addition, a significant decrease of syncytin transcript levels was observed in SOD-1 transfected cells . We then examined SOD-1 expression and activity in isolated trophoblast cells from Trisomy 21- affected placentas. SOD-1 mRNA expression ($p<0.05$), protein levels ($p<0.01$), and activity ($p<0.05$) were significantly higher in trophoblast cells isolated from Trisomy 21-affected placentas than from normal placentas. These results suggest that SOD-1 overexpression may directly impair trophoblast cell differentiation and fusion, and that of SOD-1 in Down's syndrome may be responsible, at least in part, for the failure of ST formation observed in Trisomy 21-affected placentas.

INTRODUCTION

In humans, fetal cytotrophoblasts play a key role in the embryo implantation process, and in placental development. In early pregnancy mononuclear cytotrophoblasts proliferate and invade the maternal endometrium to form the anchoring villi (1, 2). Cytotrophoblasts also fuse and differentiate into a continuous layer of multinucleated syncytiotrophoblast. This cell layer, which covers the chorionic villi, is bathed with maternal blood in the intervillous spaces from early gestation (3, 4). The syncytiotrophoblast layer plays a major role throughout pregnancy, since it is the site of numerous placental functions, including ion and nutrient exchange and the synthesis of steroid and peptide hormones required for fetal growth and development (5, 6). Some of these hormones, such as human chorionic gonadotropin (hCG), human placental lactogen (hPL) and placental growth hormone (PGH, also called growth hormone variant: GHV) are specific to pregnancy and can be used as markers of syncytium formation (7-9).

It has been established both *in vivo* and *in vitro* that the syncytiotrophoblast layer arises from the differentiation and fusion of mononuclear cytotrophoblasts. Isolated mononuclear cytotrophoblasts have been shown to aggregate and fuse together to form a non proliferative, multinucleated syncytiotrophoblast which synthesizes and secretes specific hormones required for fetal development (10, 11). This cytotrophoblast differentiation is stimulated *in vitro* by a number of factors, including EGF (12, 13), granulocyte stimulating factor (14), hCG (15, 16), glucocorticoids (17), and estradiol (18). Several studies also have shown that hypoxia inhibits cytotrophoblast differentiation and fusion (19-21). These results raise the interesting possibility that a change in cellular oxidative status may play a regulatory role in cytotrophoblast fusion and differentiation into syncytiotrophoblast.

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\cdot -}$), and hydroxyl radical ($^{\cdot}OH^{-}$) are generated in cells in response to stimulation by

various hormones, growth factors, and cytokines (22, 23). The oxygen radicals generated appear to act as second messengers in transmembrane signaling pathways to modulate cellular functions such as cell proliferation and differentiation (24, 25). Cellular oxidative status is determined by the balance between ROS production and their destruction by a variety of antioxidant enzymes. The primary antioxidant activity in the cell which regulates the level of $O_2^{\cdot -}$ and its reactive progeny are the superoxide dismutases (SODs). Mammalian cells have a mitochondrial Mn-SOD, a cytoplasmic Cu, Zn-SOD which also is found in peroxysomes, and an extracellular SOD which is a Cu, Zn-SOD that is immunologically distinct from the classical Cu, Zn-SOD (26). These metalloenzymes act to dismute generated superoxide radicals to oxygen and H_2O_2 (23). In turn, catalase, along with peroxidases such as glutathione peroxidase, catalyse the decomposition of H_2O_2 to water and oxygen (27).

Both cytosolic Cu, Zn-SOD (SOD-1) and mitochondrial Mn-SOD are expressed in human cytotrophoblasts (28, 29). Extracellular Cu, Zn-SOD appears to localize within the villous extracellular matrix around the arterioles of the placenta (30). A role for SOD-1 in placental development has been suggested by results which show reduced fertility in transgenic female mice lacking SOD-1. In SOD-1 $^{-/-}$ female mice a post-implantation embryonic loss was observed with no placental development (31). Moreover, male SOD-1-deficient drosophila are sterile, while SOD-1 deficient females exhibit markedly reduced fertility (32). However, little is known concerning a possible role for SOD-1 in human placental development. Recently, we demonstrated a modulation of SOD-1 expression and activity with *in vitro* differentiation of human villous cytotrophoblasts (33). Interestingly, we also have shown a failure of cytotrophoblast differentiation into syncytiotrophoblast in trisomy 21-affected placentas (34, 35). It has been known for sometime that SOD-1 is located on human chromosome 21, and that it is overexpressed in different trisomy 21-affected cell types (36). To better understand the role of SOD-1 in trophoblast differentiation, we have

employed an *in vitro* model of differentiation of human villous cytotrophoblast into syncytiotrophoblast to study the effect of overexpression of SOD-1 on the differentiation of these cells, and to determine the levels and activities of this enzyme in cytotrophoblast cells isolated from Trisomy 21-affected placentas which are unable to undergo normal differentiation and fusion to multinuclear syncytiotrophoblast.

EXPERIMENTAL PROCEDURES

Placental tissue collection. Term placentas were obtained after elective cesarean section from healthy mothers near term with uncomplicated pregnancies. French law allows termination of pregnancy with no gestational age limit when severe fetal abnormalities are observed. Samples of placental tissues were collected at the time of termination of pregnancy at 12-24 weeks of gestation (expressed in weeks of amenorrhea) in T21-affected pregnancies and gestational age-matched control cases. Gestational age was confirmed by ultrasound measurement of crown-rump length at 8-12 weeks of gestation. Fetal Down syndrome was diagnosed by karyotyping of amniotic fluid cells, chorionic villi or fetal blood cells. We checked that placental tissue was T21-affected by determination of DNA polymorphism markers (37). In no case was T21 due to translocation and no mosaicism was observed. Termination of pregnancy was performed in control cases affected by severe bilateral or low obstructive uropathy or major cardiac abnormalities. Fetal karyotype was normal in all controls. Placental samples were used for cytotrophoblast cell isolation or immediately frozen in liquid nitrogen.

RNA isolation and analysis. Total RNA was extracted from frozen placental samples by means of the single-step guanidinium-phenol-chloroform method described by Chomczynski and Sacchi (38) and from cultured cells following the procedure of Qiagen. Total RNA concentration was determined at 260 nm and its integrity was monitored by 1% agarose gel electrophoresis. Relative mRNA levels of the different genes were measured with the TaqMan5' nuclease fluorogenic quantitative PCR assay essentially as previously described (39). The nucleotide sequences of the primers and probes are listed in table I. Each sample was analyzed in duplicate and a calibration curve was run in parallel for each analysis. The level of transcripts of the constitutive house keeping gene product cyclophilin A was quantitatively measured in each sample to control for sample-to-sample differences in RNA concentration and quality. The PCR data are thus reported as the number of transcripts per number of cyclophylin A molecules.

Cell culture. Villous tissue was dissected free of membranes, rinsed and minced in Ca^{2+}-, Mg^{2+}-free Hank's balanced salt solution. Cytotrophoblast cells were isolated after trypsin-DNase digestion and discontinuous Percoll gradient fractionation, using a slight modification of the method of Kliman and Alsat. (10, 11). The villous sample was submitted to sequential enzymatic digestions, in a solution which contains 0.5% of powder trypsin (W/V, Difco), 5 IU/ml of DNAse I, 25 mM HEPES, 4.2 mM $MgSO_4$ and 1% (W/V) penicillin/streptomycin (Biochemical industrie) in HBSS and monitored under light microscopy. The first and/or second digestion were discarded after light microscopy analysis in order to eliminate syncytiotrophoblast fragments and the following four or five sequential digestions are kept. The cells collected during these last digestions were purified on a discontinuous gradient of percoll (5 to 70% in 5% step). The cells which have migrated in the middle layer (density 1.048-1.062 g/ml) were plated on culture dishes (10^6 cells/cm^2), attached to the dishes and 3

hours after plating they were carefully washed by three efficient washes with culture medium. Following this procedure, we have determinated that at 3 hours of culture**, 95%** of the cells isolated from normal or T21 placentas were cytokeratin 7-positive using a specific monoclonal antibody (dilution 1:200, Dako), less than 0.5% were vimentin-positive (dilution 1:200, Amersham International) and the other cells were mononucleated cells and identifed as macrophages. None of these cells were hPL-positive using a polyclonal specific antibody (dilution 1:500, Dako). Cells were plated in triplicate either on glass slides for immunocytochemistry studies or onto 60 mm culture dishes (10^6 cells/cm^2). They were cultured for 3 days as previously described (11).

Cell staining. To detect desmoplakin or E-cadherin, cultured cells were rinsed with PBS, fixed, and permeabilized in methanol at -20 °C, for 25 min. A monoclonal antidesmoplakin or E-cadherin antibody (1:400, Sigma) was then applied, followed by fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Sigma), as previously described (19).

Immunoblotting. To detect hPL, cell extracts were prepared as previously described (19) and solubilized protein (5 μg) was immunoblotted using a rabbit polyclonal antibody against hPL (1:250, Dako) and the specific band was revealed by chemiluminescence (Pierce supersignal, Interchim) after incubation with an anti-rabbit peroxidase-coupled antibody (19). To detect SOD-1, cell extracts were prepared as previously described (19) and solubilized protein (5 μg) was immunoblotted using a sheep polyclonal antibody against human SOD-1 (The binding Site Limited). The specific band was revealed by chemiluminescence (Pierce Supersignal) after incubation with an anti-sheep peroxidase-coupled antibody**.**

Hormone assay. hCG concentration was determined in culture media using the chemiluminescent immunoassay analyser ACS-180SE system (Bayer Diagnostics). Assay sensitivity was 2 mU/ml. hPL concentration was assayed (Amerlex IRMA, Amersham) in four-fold concentrated conditioned media. Assay sensitivity was 0.5 μg/ml. All values are means ± SEM of triplicate determinations.

Cu/Zn Superoxide dismutase (SOD-1) activity. Cultured trophoblast cells were washed 3 times with ice-cold phosphate buffered saline and harvested by scraping into ice-cold buffer (0.25 M sucrose, 20 mM Tris pH 7.4, 1 mM $MgCl_2$) with a cell scraper. Cells were pelleted by centrifugation at 1000 g for 5 min and then frozen at -80°C. Cell pellets were disrupted by sonication in 100 μl of 10 mM sodium phosphate buffer, pH 7. SOD-1 activity was measured as previously described (40). Briefly, xanthine-xanthine oxidase was utilized to generate an $O_2{\cdot}^-$ flux and the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to red formazan by $O_2{\cdot}^-$ was monitored at 505 nm at 30°C. The rate of INT reduction in the absence of samples was used as the reference rate (0.02 ± 0.005 absorbance/min). Each assay tube contained 1 mM EDTA, 25 μM INT, 50 μM xanthine, 1 U/ml catalase and enough xanthine oxidase to insure 100% non-inhibition, plus 50 mM (3-[cyclohexylamino]-1-propane-sulfonic acid) (CAPS) buffer, pH 10.2. All data were expressed in units of SOD activity per mg of protein.

DNA transfection. Cytotrophoblasts were isolated from placentas after elective cesarean section in healthy mothers with uncomplicated pregnancies at term. Transfection of cytotrophoblast primary cultures was performed by lipofection using the TransFast Transfection Reagent (Promega), according to a protocol adapted from Jacquemin's method (41). The transfection efficiency is 10±3% as measured by β–galactosidase assay. Briefly, 2.5

μg of pRSV-SOD-1 cloned from human syncytiotrophoblast mRNA (Pharos), or empty vector, was mixed with TransFast Reagent in a 1:1 lipid:DNA ratio. After 15-min incubation at room temperature, 2 ml of the TransFast Reagent/DNA mixture were added to each plate (60 mm), and the cells were immediately placed in the incubator for 1 hour. Cells were then overlaid with 4 ml of complete medium and returned to the incubator for 24 hours. The medium was sampled every day for 3 days to determine hCG levels. At 72 hours, the cells were harvested by scraping in the presence of 10 mM phosphate buffer and protein concentration was determined using bovine serum albumin as standard. SOD-1 mRNA and SOD-1 activity were assayed to assess transfection efficiency. Transfections were performed in triplicate with 3 different primary cell cultures and 2 different DNA preparations.

Construction of the SOD-1-GFP expression plasmid. An SOD-1-GFP gene fusion was created by amplifying a DNA product carrying the human SOD-1 gene from plasmid, pRSVSOD-1 by polymerase chain reaction. The forward (5'-GCCGATCTCGAGATGGCGA CGAAGGCCGTGTGC-3') and reverse (5'-GACCGGCCGCGGGGCGATCCCAATTACAC CACAAG-3') primers used to amplify SOD-1 incorporated XhoI and SacII restriction sites 5' and 3' to the gene, respectively. These sites were used to insert the gene into identical site within the plasmids pEGFP-N1 and pEGFP-C3 (Clonetech laboratories, Inc.). This created inframe fusions with the GFP coding region at the C-terminus (pEGFP-N1) or N-terminus (pEGFP-C3) of SOD-1.

Protein determination. Protein was determined according to Bradford's method (BioRad) using bovine serum albumin as standard.

Statistical tests. Statistical analysis was performed using the StatView F-4.5 software package (Abacus Concepts, Inc.). Values are presented as mean ± SEM. Significant differences were identified using Mann-Whitney analysis for hormonal secretions and ANOVA for transfections; $p < 0.05$ was considered significant.

RESULTS

Effect of Cu/Zn SOD-1 overexpression in normal cytotrophoblasts.

As shown previously, purified mononuclear cytotrophoblasts isolated from normal human term placenta aggregate, fuse and form a large multinucleated syncytiotrophoblast 72 hours after plating in culture (10, 11). This *in vitro* syncytiotrophoblast formation is associated with significant increases in hCGα mRNA, hCGβ mRNA, hPL mRNA, leptin and PGH mRNA levels (34). Concomitantly, hCG, hPL and leptin levels were shown to increase with time in conditioned media of the differentiated syncytiotrophoblast (34).

To determine the effect of SOD-1 overexpression on cytotrophoblast differentiation and fusion into syncytiotrophoblast, we transiently transfected isolated cytotrophoblasts with SOD-1. The purity of cytotrophoblast cell population was firstly checked by cytokeratin 7 immunostaining (Fig.1A) before transfection. As shown in Fig. 1B, in SOD-1 transfected cells, both SOD-1 mRNA and SOD-1 enzymatic activity were found to be elevated. After transfection of these primary cultures, SOD-1 mRNA levels were increased about 5 fold while SOD-1 activity was increased by approximately 30%. No increase in SOD-1 mRNA or SOD-1 activity was detected in control cells transfected with the empty vector. Western blot analysis showed that SOD-1 protein levels also were significally higher (45% and $p \leq 0.001$) in transfected cells relative to control cells transfected with the empty vector (Fig.1C).

With differentiation and fusion of cytotrophoblasts into syncytiotrophoblast, there is an increase in the mRNA transcript levels of the placental hormones, hCGα, hCGβ, hPL and placental GH. The appearance of these hormones can be used as markers of syncytium formation. As shown in Fig.2A, the mRNA levels of these marker hormones were significantly decreased in cells overexpressing SOD-1 compared to control cells transfected by the empty vector. Further, the overexpression of SOD-1 in normal cytotrophoblasts also resulted in a significant ($p \leq 0.0001$) decrease in hCG secretion compared to control cells transfected by the empty vector (Fig.2B). In other studies, hPL production could be detected in the culture medium of SOD-1 transfected cells (data not shown).

A recent study has shown that a retroviral envelope glycoprotein HERV-W, also called syncytin is directly implicated in the trophoblastic fusion process (42). Indeed, the expression of recombinant syncytin in a variety of cell types was shown to induce the formation of giant syncytia. Further, the fusion of a human trophoblastic cell line expressing endogenous syncytin was found to be inhibited by an anti-syncytin antiserum. These data indicate that syncytin may mediate placental cytotrophoblast fusion. Thus, we determinated the expression of syncytin mRNA in cytotrophoblasts by real-time quantitative RT-PCR (Fig.3). First, we confirmed with our primary culture of human cytotrophoblast model that there is a significant increase ($p \leq 0.0002$) in syncytin mRNA levels during the cytotrophoblast differentiation and fusion (Fig.3A). In SOD-1 transfected cells, syncytin mRNA expression was found to be decreased compared to control cells transfected with the empty vector (Fig.3B).

Overexpression of GFP-tagged SOD-1 in normal cytotrophoblasts inhibits cell fusion.

To further demonstrate that overexpression of SOD-1 may be implicated in the failure of cytotrophoblasts to fuse and differentiate, PCR products of SOD-1 were produced and then cloned into the pEGFP-N1 vector as described in materials and methods. This expression

vector then was used to transfect normal human cytotrophoblasts to overexpress GFP-tagged SOD-1. Cells overexpressing GFP-SOD-1 can be recognized within the cell population by the presence of green fluorescence due to the presence of the GFP tag. It was observed that cytotrophoblasts overexpressing GFP-tagged SOD-1 remained mononucleated and aggregated, as visualized by the detection of desmoplakin (Fig.4). In contrast, cells on the same dish which were not overexpressing GFP-SOD-1 (absence of green fluorescence) fused to a multinucleated syncytium. Similar results were obtained using expression vectors containing GFP fused to either the C-terminus or N-terminus of SOD-1 (data not shown). Moreover, the GFP-tagged SOD-1 fusion protein was shown to retain catalytic activity, as determinated by assay of increased SOD-1 activity (data not shown).

SOD-1 mRNA and protein levels in normal and Trisomy 21-affected placentas.

We recently observed that villous cytotrophoblasts isolated from Trisomy 21- affected placentas either do not, or poorly, differentiate and fuse into multinucleated syncytiotrophoblast (34). Due to the known location of the human SOD-1 gene on chromosome 21 (43), we measured the SOD-1 mRNA and protein levels in total tissue extracts prepared from 5 normal and 7 trisomy 21-affected placentas matched for gestational age. Figure 5, panel A, shows SOD-1 mRNA levels in these total tissue extracts, normalized to pleiotropin (PO), cyclophilin A (PPIA), and cytokeratin 7 (KRT7) mRNA expression as reference markers. No significant difference in SOD-1 mRNA transcript levels was found between Trisomy 21 affected and normal total tissue extracts, regardless of the reference gene used. Western blotting (Fig.5B) showed no difference in SOD-1 protein levels between the normal and abnormal tissues. These results were consistent with the similar SOD-1 mRNA levels in normal and Trisomy 21-affected placentas.

SOD-1 mRNA and protein levels in trophoblast cells isolated from normal and Trisomy 21-affected placentas.

Since cytotrophoblasts constitute a small percentage of the heterogeneous cell population found in placentas, we wanted to determine directly if SOD-1 might be increased in isolated population of T21 cytotrophoblasts. Thus, cytotrophoblast cells were purified and isolated from gestational age-matched normal and Trisomy 21-affected placentas as described in Methods. As shown in Figure 6 (panel A), SOD-1 mRNA was found in T21 trophoblast cells at a level about 1.5 times higher than in normal trophoblast cells. This increase in SOD-1 mRNA expression was statistically significant ($p<0.05$) and highly reproducible. SOD-1 protein levels and catalytic activity also were determined in both normal and Trisomy 21-affected trophoblast cells (panels B and C). Again, the SOD-1 protein levels ($p<0.01$) and enzymatic activity ($p<0.05$) were significantly higher in Trisomy 21 cells than in normal trophoblast cells, in keeping with the observed elevation of mRNA levels found in T21 cytotrophoblasts. These results establish and confirm that cells of individuals with this genetic disease, including cytotrophoblasts, have elevated levels of SOD-1 protein and catalytic activity. Further these cytotrophoblasts isolated from Trisomy 21-affected placenta poorly differentiate into syncytiotrophoblasts.

DISCUSSION

Few human cell types can fuse together and differentiate into multinucleated syncytia. This process is involved in the formation of myotubes (44), osteoclasts (45) and syncytiotrophoblast (3). The syncytiotrophoblast is the primary site of several placental functions, including nutrient exchanges, metabolism and steroid and peptide hormone

synthesis, which are required for fetal growth and development (5, 6, 46). Despite a common morphological differentiation process, the three cell types which are able to differentiate into a syncytium differ notably. Syncytiotrophoblast *in situ* maintains a strong polarity, with microvilli on the apical membrane, while myotubes do not exhibit morphological polarity. The myoblast-myotube transition first requires the withdrawal of myoblasts from the cell cycle to G_0, while cytotrophoblasts which fuse to create the syncytiotrophoblast already are essentially in G_0 (10). In contrast to syncytiotrophoblast, osteoclasts have major locomotor activity.

The cell-cell fusion process involved in syncytiotrophoblast formation is poorly understood (47). *In vitro* studies have established that soluble factors such as EGF (12), parathyroid hormone (11) and hCG (15) activate different intracellular signaling pathways to stimulate the differentiation of villous cytotrophoblasts into syncytiotrophoblast. The cellular processes leading to syncytial formation are associated with a concomitant increase in the intracellular level of cyclic AMP (48) (49). This elevation in cyclic AMP levels is required for synthesis of numerous specific trophoblast proteins and hormones (see for review (50). We also have reported a direct role for cyclic AMP dependent protein kinases in simulating cytotrophoblast fusion (51). On the other hand, we and others have observed that cytotrophoblast fusion and differentiation is inhibited by hypoxia (19-21). Similarly the histological abnormalities of term placentas in pregnancy associated with underperfusion and hypoxia are characterized by cytotrophoblast prominence and abnormalities in syncytiotrophoblast differentiation (52, 53). This suggests that the oxidative state of the cytotrophoblast may be a key element in regulating differentiation into syncytium, and points to a direct role for oxygen derived free radicals in the modulation of cell fusion.

Cu/Zn superoxide dismutase (SOD-1) is a cytoplasmic enzyme which protects cells from oxygen-derived free radicals (26). SOD-1 transforms the superoxide anion $O_2^{\cdot -}$ into

hydrogen peroxide, which is then converted to water by peroxisomal catalase and glutathione peroxidase (GPx). This two-step process eliminates H_2O_2 and other reactive oxygen species that could otherwise interact with macromolecules such as DNA, proteins and lipids, to alter their structure and function. However, any alteration of the balance between the first and second step may induce an oxydative stress related to the misregulation of H_2O_2 production.

The SOD-1 gene is located on human chromosome 21 (43). The activity of this enzyme is increased by about 50% in the red blood cells (54), platelets (55), lymphocytes, polymorphonuclear granulocytes and fibroblasts (56) of individuals with Down's syndrome (Trisomy 21). In this study, we first established that SOD-1 mRNA, protein and activity were present in isolated normal human trophoblast cells, confirming and extending previous reports, based on RT-PCR, differential display and immunostaining (12, 28, 57). It then was shown that SOD-1 expression and activity in purified trophoblast cells isolated from Trisomy 21-affected placentas were about 50% higher than in normal trophoblasts, in keeping with a gene dosage effect. This increase in SOD-1 activity in cytotrophoblasts isolated from Trisomy 21-affected placenta is associated with a defect of fusion and syncytiotrophoblast formation. These *in vitro* data are in agreement with recent data reporting histomorphological features of chorionic villi in Trisomy 21-affected pregnancies, showing increased double layer proliferative trophoblasts (58). This suggests that overexpression of SOD-1 leads to increased oxidative stress and trophoblast injury tending to stimulate proliferation and to decrease differentiation as observed in other pathological conditions.

However, when SOD-1 expression was compared in total tissue extracts from Trisomy 21-affected placentas and normal controls matched for gestational age, no significant differences was found in SOD-1 transcript or protein levels. This finding appears to be due to the heterogeneous composition of the whole placenta. Total placenta extracts contain material of fetal origin, including fibroblasts, endothelial cells, and trophoblast cells. It also contains

material of maternal origin, including red blood cells, along with endothelial and decidual cells; Therefore, SOD-1 levels in total placental extracts reflect the SOD-1 expression in various cells of both fetal and maternal origin. SOD-1 is known to be highly expressed in maternal red blood cells and decidual cells (59). Since cytotrophblasts make up only a small percentage of the heterogeneous cell population found in placentas, we were unable to detect an elevation in SOD-1 levels in total tissue extract from the Trisomy 21-affected placentas.

In this study, experimental evidence suggests that an alteration in the oxydative state of human trophoblast cells related to overexpression of SOD-1 appears to be associated with a failure of differentiation and fusion into syncytiotrophoblast. This was illustrated by the inability of cells overexpressing SOD-1 to undergo cell-cell fusion. Indeed, we observed that primary normal human trophoblasts overexpressing SOD-1 tagged with green fluorescent protein remained mononucleated and aggregated, as visualised by the detection of desmoplakin. In contrast, cells on the same dish which did not expressed SOD-1-GFP were able to fuse to a multinucleated syncytium. The inability of cells overexpressing SOD-1 to fuse and differentiate into syncytiotrophoblast was associated with a significant decrease in the transcipt levels of genes encoding for pregnancy specific hormones such as hCG, hPL and placental growth hormone. These hormones are specifically expressed only by the differentiated syncytiotrophoblast. It should be noticed that this significant decrease in syncytiotrophoblast hormonal markers expression was in contrast with the relatively low transfection efficiency of these primary trophoblast cultures. Therefore it can not be excluded that by cell-cell interaction or paracrine effects, one transfected cell may affect different non transfected neibourhing cells. Indeed, modulation of the oxidative status of one trophoblast cell overexpressing SOD-1, may induce for exemple cytokine secretions involved in trophoblast differentiation.

Other evidence has suggested that endogenous retroviral gene expression also may be involved in the mediating cell fusion. Indeed, high expression of retrovirus is one of the characteristic of human syncytiotrophoblast (60, 61). This observation of retroviral particles in placenta, along with the presence of fused placental cells morphologically reminiscent of virally-induced syncytia, led to the proposal that an ancient retroviral infection may have been a pivotal event in mammalian evolution (61). Recently, syncytin gene expression, which codes for a retroviral envelop protein, was shown to be increased, and required for trophoblastic cell fusion (42). In this communication, we confirmed by real-time PCR that the transcript levels of syncytin increased with the differentiation and fusion of cytotrophoblasts into syncytiotrophoblast. Further, it was shown that impaired cell fusion related to overexpression of SOD-1 was associated with a decrease in the transcript level of syncytin.

Individuals with Down's syndrome appear to exhibit increased oxidative stress (62, 63). They develop Alzheimer-like neuronal changes by the third or fourth decade of life, the incidence of autoimmune diseases and cataracts is significantly increased (64), and the overall aging process is accelerated (65). In addition, evidence that SOD-1 may be involved in the pathophysiology of Down's syndrome includes the demonstration that SOD-1 gene overexpression can impair neurotransmitter transport and alter neuromuscular junctions (66, 67). Here evidence is presented which indicates that elevated SOD-1 levels may play a critical role in modulating the cell differentiation, especially the cell-cell fusion, process involved in human syncytiotrophoblast formation. Results presented in this communication also clearly demonstrate a relationship between elevated SOD-1 levels in human cytotrophoblasts and the decreased production of pregnancy-specific hormones found with differentiation to syncytiotrophoblast. Taken together, the results presented here suggest that the elevated level of SOD-1 noted in Trisomy 21 may be responsible, at least in part, for the

failure of cytotrophoblasts to fuse and from multinucleated syncytiotrophoblast as noted in Down syndrome (34, 35).

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Characterization of SOD-1 overexpression in normal cytotrophoblast cells.

Panel A: Cytokeratin 7 immunodetection after 24 hours of culture of trophoblast cells isolated from normal placenta. Positive immunofluorescence staining is specific of cytotrophoblast cells.

Panel B: Transfection experiments. Cytotrophoblast cells isolated from normal placenta were transfected with an empty plasmid used as control (Control), or with a SOD-1 expression vector (SOD-1). Data are expressed as levels of SOD-1 mRNA normalized by PPIA mRNA (Peptidylprolyl isomerase A, cyclophilin A). SOD-1 activity in these cells is expressed as IU/mg of protein. The results presented are expressed as the mean ± SEM of 3 culture dishes.

Panel C: SOD-1 protein levels after transfection were determined by western blotting with a sheep polyclonal antibody to SOD-1. The autoradiogram (upper panel) shows a specific band of 17 kDa in normal cytotrophoblasts transfected either with an empty plasmid (Control, 3 separate dishes), or with a SOD-1 expression vector (SOD-1, 3 separate dishes). The lower histogram represents densitometry quantification of the autoradiograms (mean ± SEM of 3 culture dishes).

The figure represents one of three experiments. ***: $p \leq 0.001$.

Figure 2: SOD-1 overexpression in normal cytotrophoblasts inhibits the increase in placenta hormone production noted with differentiation.

Panel A: hCGα, hCGβ, PGH, hPL and leptin mRNA levels determined, by quantitaitve real-time PCR, in normal cytotrophoblasts transfected either with an empty plasmid used as control (Control), or with a SOD-1 expression vector (SOD-1). These assays were carried out

72h after plating. Values are the levels of each hormonal mRNA normalized to the level of PPIA mRNA.

***Panel B*:** levels of hCG secreted into the culture medium 72 hours after transfection determined by chemiluminescent immunoassay. Normal cytotrophoblasts were tranfected either with an empty plasmid used as control (Control), or with a SOD-1 expression vector (SOD-1). Data are the mean values for 3 separate dishes ± SEM.

The figure illustrates one representative experiment of 3 performed. *: $p \leq 0.05$; ***: $p \leq 0.001$.

Figure 3: SOD-1 overexpression in normal cytotrophoblasts blocks the increase of syncytin mRNA expression noted with differentiation.

Panel A: Syncytin mRNA levels are mesured by real-time quantitative RT-PCR during normal cytotrophoblast differentiation and fusion.

Panel B: Syncytin mRNA levels were measured by real-time quantitative RT-PCR in normal cytotrophoblasts after transfection either with an empty plasmid (Control), or with a SOD-1 expression vector (SOD-1). Syncytin mRNA levels are normalized to PPIA mRNA levels (mean ± SEM); ***: $p \leq 0.001$. These assays were carried out 72h after plating.

Figure 4: Overexpression of GFP-SOD-1 protein in normal cytotrophoblasts inhibits cell fusion.

Cells overexpressing GFP-SOD-1 can be detected by the appearence of green fluorescence. The cells expressing GFP-SOD-1 remain aggregated and do not fuse, as determinated by the presence of desmoplakine (red fluorescence). In the same dish, cells which are not expressing the GFP-SOD-1 chimera protein were able to differentiate and fuse to a multinucleated syncytium as determinated by the absence of desmoplakine. Nuclei were labelled with DAPI (blue fluorescence).

Figure 5: SOD-1 mRNA and protein expression in total tissue samples obtained from normal (N) and Trisomy 21-affected placentas (T21).

Panel A: SOD-1 mRNA levels determined, by real-time quantitative PCR are normalized to PO, PPIA and KRT7 mRNA levels (mean ± SEM). SOD-1 mRNA levels were determined in 5 normal placentas and 7 Trisomy 21-affected placentas.

Panel B: SOD-1 protein levels determined by western blotting with a sheep polyclonal antibody to SOD-1. This autoradiogram shows a specific band of 17 kDa in normal (N) and Trisomy 21-affected placentas (T21). The lower histogram represents densitometry quantification of the autoradiograms (N: n=5; T21: n=7).

Figure 6: SOD-1 mRNA and protein levels, and catalytic activity, in purified trophoblasts prepared from normal (N) and Trisomy 21-affected placentas (T21).

Panel A: SOD-1 mRNA levels determined, by real-time quantitative PCR, are normalized to PPIA mRNA levels (mean ± SEM). SOD-1 mRNA levels were determined in isolated trophoblasts prepared from 5 normal and 7 Trisomy 21-affected placentas. *: $p \leq 0.05$

Panel B: Human SOD-1 protein levels determined by western blotting with a sheep polyclonal antibody to SOD-1. The autoradiogram shows a specific band at 17 Kda in trophoblasts isolated from normal (N) and Trisomy 21-affected placentas (T21). The histogram represents densitometric quantification of the autoradiograms (N: n=5; T21: n=7).

Panel C: Human SOD-1 activity is expressed in IU/mg protein. The results are expressed as means ± SEM. *: $p \leq 0.05$; **: $p \leq 0.01$.

Figure 1

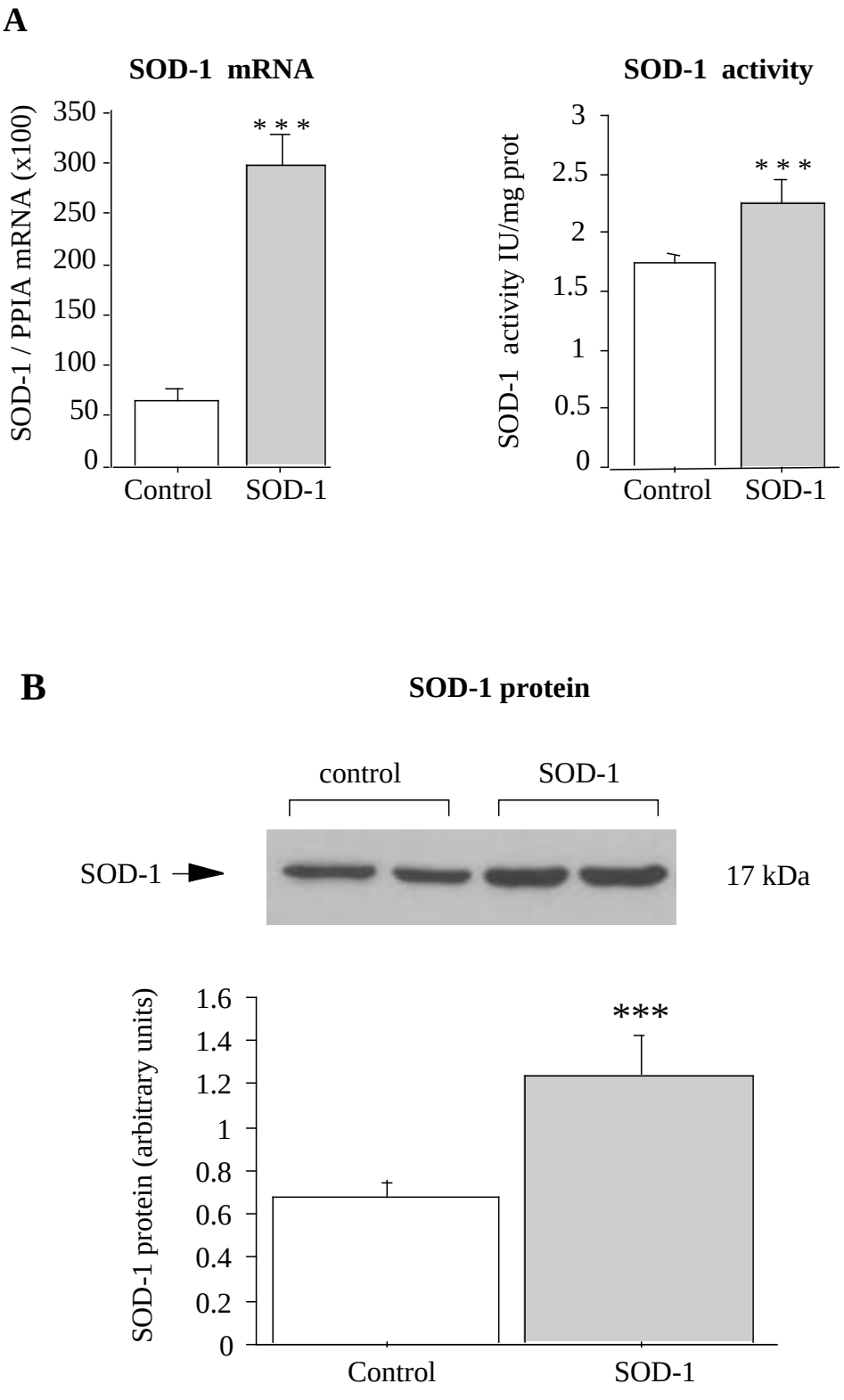


Figure 2

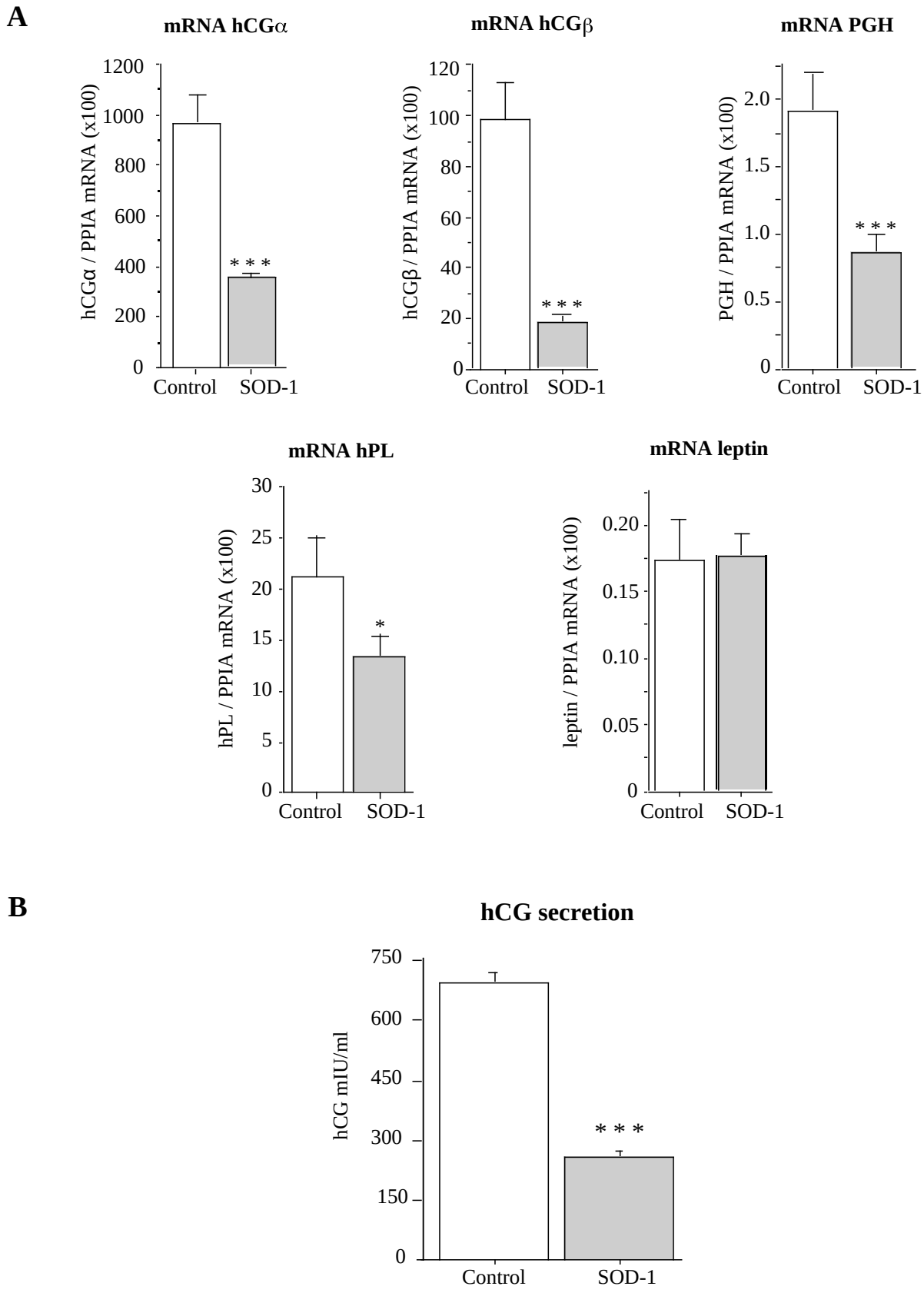
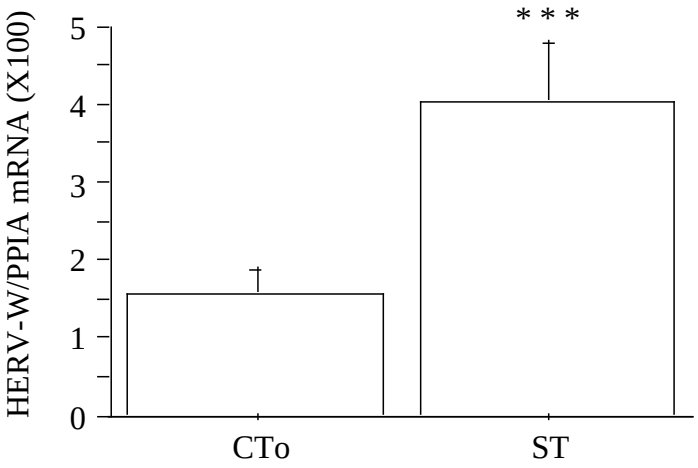


Figure 3

A



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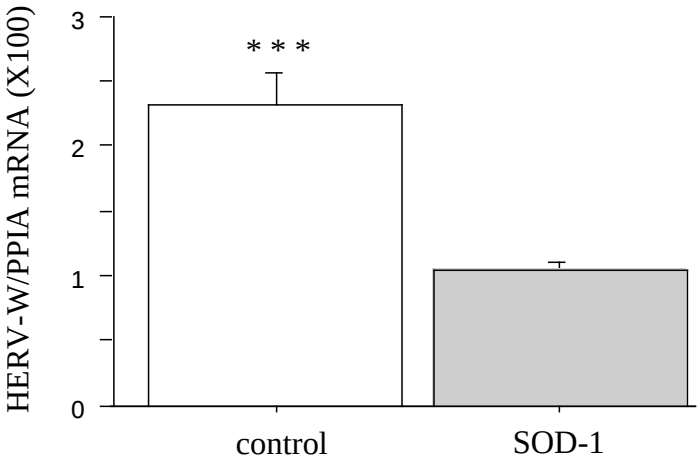


Figure 4

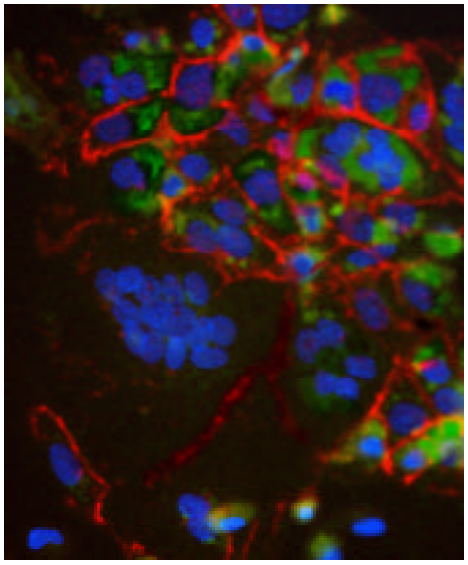


Figure 5

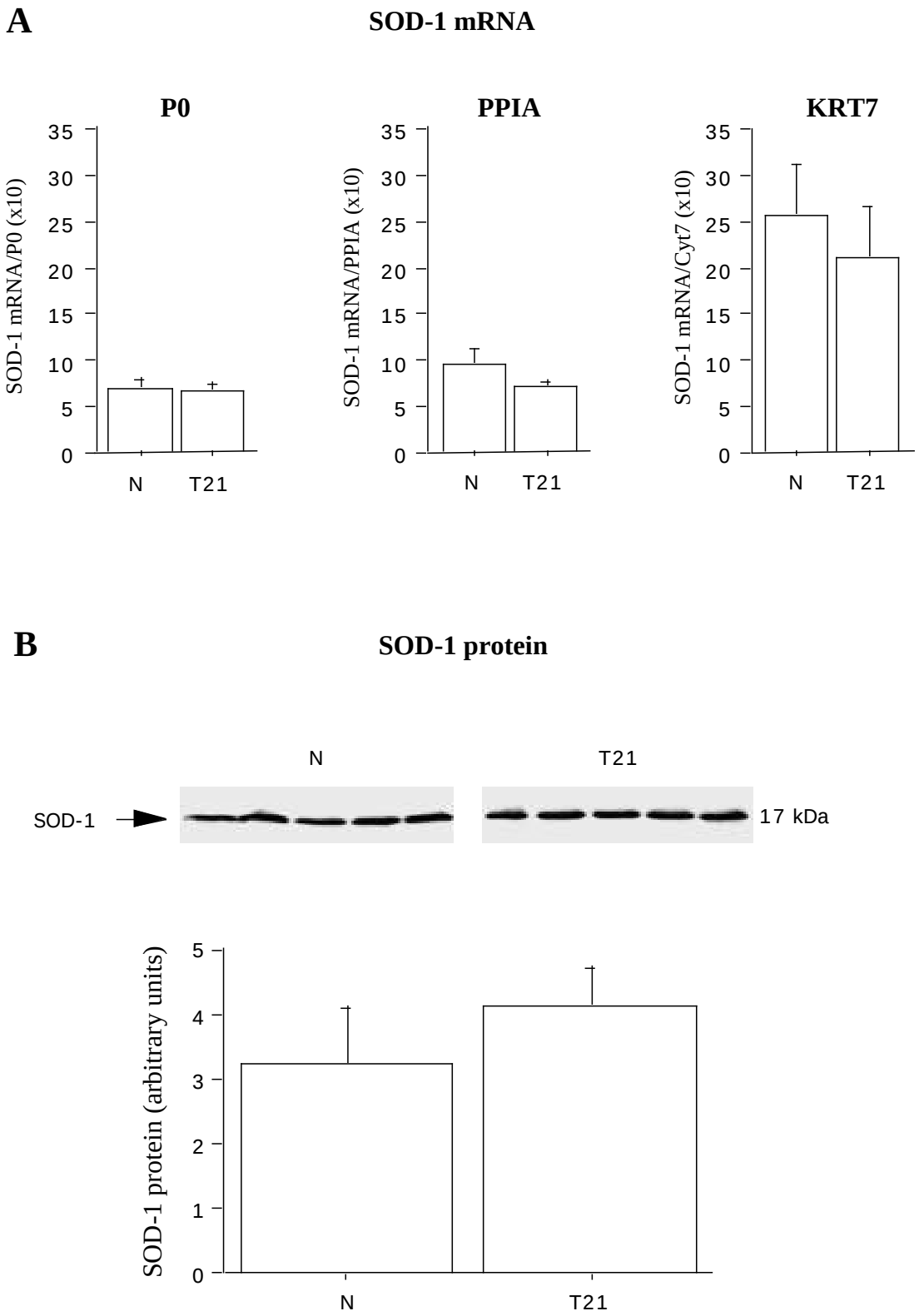
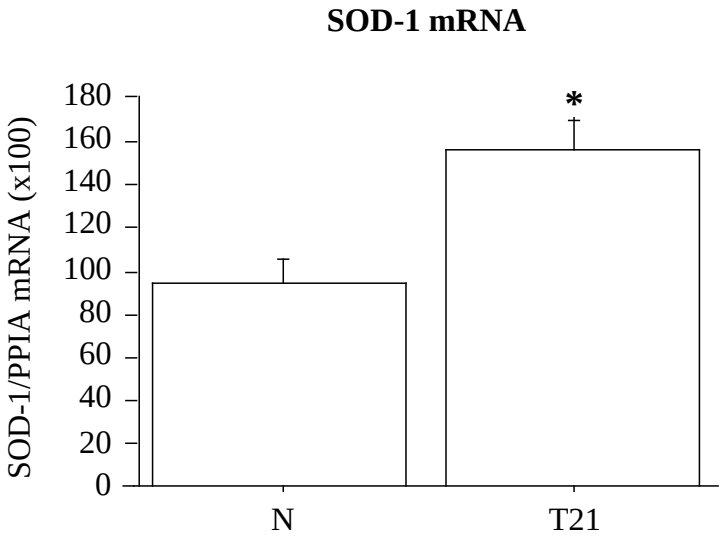


Figure 6

A



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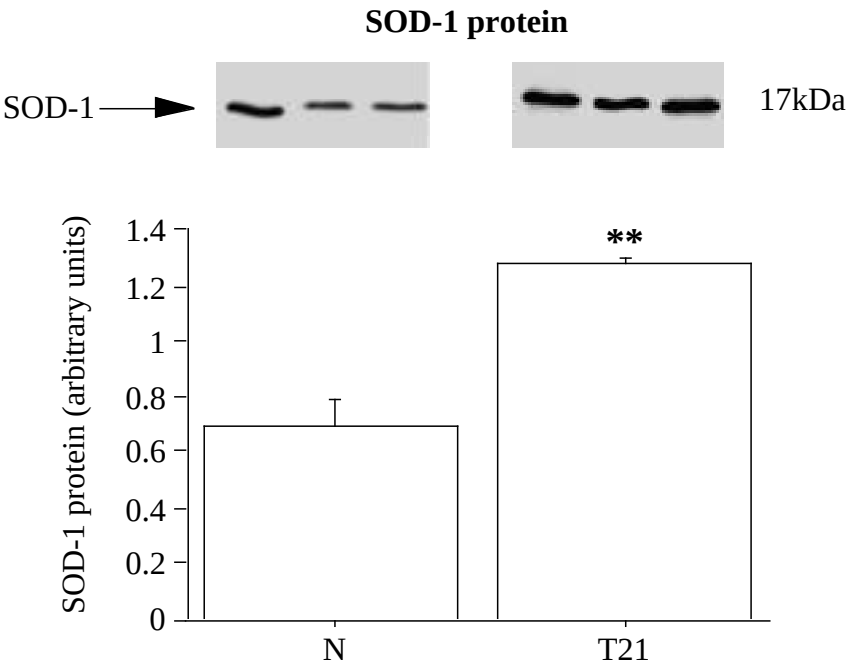

C

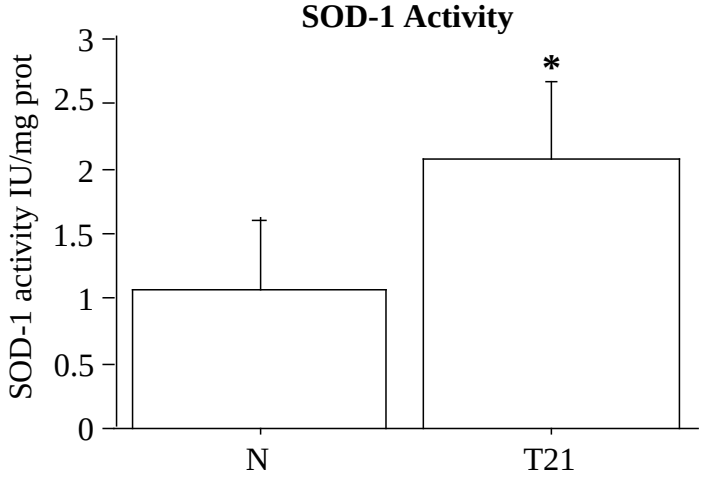

Table 1: Characteristics of the PCR assays used in this study.

genes	primers and probes		GenBank accession number	proteins
CGA	(+) TCCCACTCCACTAAGGTCCAA (-) CCCCATTACTGTGACCCTGTT FAM-CACAGCAAGTGGACTCTGAGGTGACG-TAMRA	106 bp	V00518	hCGα
CGB	(+) GCTACTGCCCCACCATGACC (-) ATGGACTCGAAGCGCACATC FAM-CCTGCCTCAGGTGGTGTGCAACTACC-TAMRA	94 bp	J00117	hCGβ
CSA	(+) GCATGACTCCCAGACCTCCTT (-) TGCGGAGCAGCTCTAGATTGG FAM-TTTCTGTTGCGTTTCCTCCATGTTGG-TAMRA	157 bp	J03071	hPL
GHV	(+) AGAACCCCCAGACCTCCCT (-) TGCGGAGCAGCTCTAGGTTAG FAM-TTTCTGCTGCGTTTTCACCCTGTTG-TAMRA	96 bp	J03071	PGH
LEPTIN	(+) ACATTTCACACACGCAGTCAGT (-) CCATCTTGGATAAGGTCAGGAT FAM-TGGAGCCCAGGAATGAAGTCCAAA-TAMRA	96 bp	U18915	leptin
KRT7	(+) GGACATCGAGATCGCCACCT (-) ACCGCCACTGCTACTGCCA FAM-TCACGGCTCCCACTCCATCTC-TAMRA	171 bp	X03212	cytokeratin 7
PTN	(+) GAGATGTAAGATCCCCTGCAACT (-) CTCGCTTCAGACTTCCAGTTCT FAM-AGCAATTTGGCGCGGAGTGCAA-TAMRA	125 bp	M57399	pleiotropin
PPIA	(+) GTCAACCCCACCGTGTTCTT (-) CTGCTGTCTTTGGGACCTTGT FAM-AGCTCAAAGGAGACGCGGCCCA-TAMRA	97 bp	Y00052	cyclophilin A
HERV-W	(+) CGGACATCCAAAGTGATACATCCT (-) TGATGTATCCAAGACTCCACTCCA	100 bp	/	syncytin
SOD-1	(+) CTGAAGGCCTGCATGGATTC (-) CCAAGTCTCCAACATGCCTCTC FAM- TCATCCTTTGGCCCACCGTGTTT -TAMRA	138 pb	X02317	CuZn superoxide dismutase